Variability of Hormonal Stress Markers Collected from a Managed Dolphin Population

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LONG-TERM GOALS

Quantifying physiological indicators of stress in wild marine mammals and the interrelationships between different stress markers can be used to estimate the impact of anthropogenic stressors on marine mammal populations. The United States Navy, as part of its environmental stewardship, can utilize stress markers to assess the acute and chronic impacts that its actions might have on marine mammals. This approach would permit better mitigation of potential impacts and ensure that Navy activities do not come at a deleterious cost to marine mammal populations.

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OBJECTIVES

The objectives of this effort are to: 1) determine the variation in corticosteroid hormones, thyroid hormones, and catecholamines within a dolphin population relative to seasonality, time of day, gender, age and reproductive state; 2) assess relationships between serum corticosteroid levels and levels found in other matrices (i.e. biological samples), including feces, saliva, and blubber; 3) and to perform adrenocorticotropic hormone (ACTH) and thyrotropin-releasing hormone (TRH) challenges to characterize the activation of the hypothalamic-pituitary-adrenal (HPA) and hypothalamic-pituitary-thyroid (HPT) axes across multiple matrices, respectively.

APPROACH

Task 1 – Seasonal variations in hormones across multiple matrices
Regular sampling from different matrices (e.g. blubber, blood, feces) will be collected from the U.S. Navy Marine Mammal Program (MMP) dolphin population over the course of a year. Subject dolphins will be split into categories based upon age: 5-15 years, 16-25 years, and 26-35 years. Each animal will be sampled bi-weekly throughout the year for blood and feces. A subset of animals will be selected for blubber biopsies, which will be conducted every month.

Blood samples will be collected from dolphins through their voluntary participation. Blood collections will be made from the ventral fluke from the arteriovenous plexus and collections will be made between 0700-1000. Fecal samples will be collected by use of a suction catheter inserted into the anus of the dolphin. Fecal samples will be collected through voluntary cooperation and will be performed the day after the blood collection. Bimonthly blubber biopsies will be collected with a 16g or 18g tissue biopsy needle and the condition and healing of the animal biopsied will be monitored daily following the procedure.

Serum samples will be processed for adrenocorticosteroids, catecholamines, and thyroid hormones via radioimmunoassay (RIA). Radioimmunoassay methods have previously been validated for cortisol and aldosterone in this species (Houser et al., 2011). Parallel processing of serum catecholamines will be performed via high-performance liquid chromatography (HPLC) to assess variability in the measurement methods available for these hormones..

Metabolites of cortisol, aldosterone and thyroid hormone will be extracted from fecal samples and measured via RIA using established techniques (Wasser et al., 2010). Thyroid measures will focus on T3 because I^{131} ingestion studies showed that thyroid hormone was excreted in feces almost entirely as T3 with very little T4 in two domestic dogs, and similarly only immunoreactive T3 was primarily found in killer whale feces with lesser amounts of T4 (Wasser et al., 2010).

A multi-step biphasic organic solvent extraction will be used to isolate the corticosteroids from the blubber tissue (Kellar et al., 2009). The hormones will be measured using a commercially available enzyme immunoassay (EIA) and parallel processing via HPLC will be used to verify method performance.

Task 2 – Diurnal variation in hormone production
Hormones will be assessed for diurnal variation during the second year of the study with the goal of assessing changes that occur between dawn and dusk. Ten dolphins will be selected for repeat testing throughout the year. Blood samples will be collected from the dolphins at biweekly intervals via

voluntary venipuncture of the arteriovenous plexus on the ventral fluke. Paired or triplicate blood samples will be collected on the day of sampling. The initial samples will be collected first thing in the morning (~0700) and the second either at noon or in the late in the afternoon (~1700). Blood samples will be processed via RIA and HPLC as described under Task 1. Similar analyses will be conducted on serially collected scat of these 10 individuals over the same 24 hr period and a second 24 hr period one week later when not being sampled for blood.

Task 3 – Adrenocortical sensitivity

Adrenocortical sensitivity and the relationship between activation of the HPA axis and reflection of this activation in serum and other matrices will be determined. The information from this assessment will allow researchers to better understand the temporal and quantitative relationship between hormones measured in matrices likely to be collected from wild animals, namely feces and blubber, and that circulating in the blood stream.

Three animals will be selected for determining the appropriate dose of ACTH required to sufficiently elevate the corticosteroids in serum and other matrices. ACTH slow-release gel will be intramuscularly implanted to permit time-controlled and sustained release of ACTH. Implantation of the gel will be performed by MMP or NMMF veterinarians and the animal will be monitored for several hours following the injection. Repeat blood samples will be taken over a course of several days to determine the relationship between the time course of serum corticostroid increase and the ACTH dose administered. Based upon results of the pilot study, a schedule will be determined for the collection of samples from other matrices (feces and blubber) that will be tested during the second and third years of the study. Five dolphins will be selected during for ACTH challenges following conclusion of the pilot. For each of the dolphins, blood, feces and blubber samples will be collected according to the sampling schedule determined during the first year of the study.

A pilot study will also be conducted in which a dolphin is fed fish containing cortisol pellets. For the first pilot study, 10 mg of cortisol will be fed to the dolphin in fish at six hour intervals over multiple days to attempt to raise and maintain the serum cortisol levels. Voluntary blood samples will be collected across multiple days and different time frames to determine if serum cortisol levels are elevated and sustained. Based on the results, the pilot will either be repeated with an increase in the cortisol dosage or frequency of delivery, as determined in consultation with the attending veterinarian. Provided the procedure adequately raises cortisol levels, the process will be repeated with five bottlenose dolphins and will be coupled to blubber biopsies so cortisol deposition in the blubber can be assessed.

Task 4 – Thyroid challenges

Thyroid hormones (thyroxin, T4 and triiodothyronine, T3) are released from the thyroid gland and are responsible for regulating the metabolism of an animal and affect the activity of other stress hormones via permissiveness. Thyroxin is the more abundant of the two thyroid hormones in circulation and the metabolic parent hormone. However, the bioactive form is largely T3, which is roughly eight times more potent than T4 (Tomasi 1991). Thyroid hormone production is known to be affected by stress, which can lead to conditions of both hypo- and hyperthyroidism. Persistent elevated or diminished levels of these hormones are known to lead to pathophysiological conditions that can ultimately impact important life history functions.

Thyroid hormones are produced in response to the presence of thyroid stimulating hormone (TSH), which is a peptide hormone produced in the anterior pituitary gland. Thyroid stimulating hormone is

itself produced in response to the action of thyrotopin-releasing hormone (TRH), which is produced in the hypothalamus. Assessing the responsiveness of the production pathway to acute elevations of TRH, i.e. a hormone challenge, is one means by which pathway responsiveness and activity of TRH can be quantified at different levels of the synthesis pathway.

Three dolphins will be given an exploratory TRH challenge to determine the optimal dosing and sampling schedule. A pre-test blood draw will be collected from the dolphin while it is in its enclosure. The dolphin will then be removed from the water to a location on the pier that is deemed suitable for the procedure by the attending veterinarian. A bolus injection of of TRH will be intravenously administered via the venous plexus of the fluke, or an alternative route as deemed necessary by the attending veterinarian. Blood samples will then be collected every 15 minutes for a period of 4 hours. Following completion of the sampling period, the dolphin will be returned to its enclosure. The dolphin will be monitored following the procedure for a period of time to be determined by the attending veterinarian. Dosages of TRH will be adjusted for the second and third animal in the pilot study following analysis of the blood samples collected with the first challenge. Following the pilot study, eight individuals will be submitted to TRH challenges. Baseline blood and fecal samples will be collected prior to the first injection and blood collections will be performed as described for the pilot studies (with minor modifications to the blood draw schedule as determined by the pilot study results). Fecal samples will be collected for 96 hrs following injection.

WORK COMPLETED

Task 1 – Seasonal variation in stress hormones
A group of 30 bottlenose dolphins were identified from within the U.S. Navy Marine Mammal Program's animal population that could provide biweekly blood samples over a period of a year. One of the animals was removed from the study after several months and was replaced by a comparably aged animal of the same gender. The following distribution of animals was obtained:

Age (yrs)	Male	Female
5-15	6	4
16-25	3	3
25+	7	7

Each of the dolphins was scheduled for a voluntary blood sample on a biweekly schedule with an attempt at a fecal collection the following day. Four animals were identified for monthly blubber biopsies to be collected on the same day following the blood collections.

A total of 735 blood collections were made out of a total of 778 possible draws (~94% success rate). A total of 638 matched fecal samples were collected such that 87% of the blood samples had matched fecal comparisons. In nearly all instances, sufficient blood was collected to perform analyses for corticosteroids, thyroid hormones, and catecholamines. Three of the female dolphins in the study became pregnant during the course of the study. Excepting the one dolphin that birthed, the pregnancies did not significantly interfere with sample collection. The dolphin that gave birth was removed from the study for several weeks to permit mother-calf bonding but was returned to the study before its conclusion.

Initial blubber biopsies were collected with a 18g BioPince biopsy needle but this was changed to a 16g biopsy needle for most of the biopsies. Biopsies were collected approximately 12-14 cm below the posterior insertion of the dorsal fins. Two or three biopsies were taken each sample period to ensure that sufficient blubber was obtained for analysis. Over the course of the year, biopsies were collected from four of the study dolphins on a bimonthly schedule. All but one of the samples were collected for a total of 47 samples. The single missed sample was due to animal illness. Samples were collected on the same day as blood collections to ensure comparisons between the matrices could be completed. The sample size was expanded to four animals in the second month. All samples were stored at -80° C until they could be processed.

Ten of the study dolphins concurrently participated in an immunological study as part of a collaborative effort with Dr. Pat Fair. The objective of the study was to compare immunological profiles of dolphins that were wild (coastal Charleston/Indian River) and those that were semi-domestic living in open-ocean (Navy dolphins) and contained systems (Georgia Aquarium). On a bimonthly schedule, eleven additional blood tubes and saliva samples were collected in conjunction with the normal sample collection. All samples collected as part of the collaborative effort were shipped to project collaborators for analysis.

Blubber samples for the first half of the year were delieverd to N. Kellar for processing. All plasma samples for HPLC analysis were delivered to T. Romano. All fecal samples were delivered to S. Wasser for corticosteroid analysis.

RESULTS

The success of the year-long, biweekly sample collection demonstrates the ability to use voluntary blood sampling to obtain relatively unstressed baseline data. Initial processing indicates that dolphins under human care produce low levels of corticosteroids. In many instances, levels are sufficiently low that alternative means of processing will need to be employed to accurately assess circulating levels.

Initial results of circulating hormones indicated that approximately 1/3 of the dolphins, all males, were affected by a pharmaceutical they were taking. The compound, megesterol acetate (MegAce), is commonly used in the marine mammal industry to control the rutting behavior of male dolphins. Daily doses as low as 20 mg were found to halt ACTH production and subsequent cortisol and aldosterone release, a condition that is physiologically similar to Addison's disease. The long-term impact of this condition is unknown. Due to its widespread use, this incidental finding has significant ramifications for the welfare of dolphins under human care.

IMPACT/APPLICATIONS

The ability to identify stress markers relative to monitoring the health of marine mammal populations is critical to understanding the impact of anthropogenic activities upon those populations. The baseline characterization of stress marker variation in dolphins as a function of seasonality, gender, age, and reproductive status is important to assessing measurements made in wild dolphins. Information on levels and dynamics of stress markers between different matrices will provide better estimates of the overall condition of marine mammals sampled in the wild from either blubber biopsies or fecal collections. In addition, an understanding of the function of the HPA and HPT axis will provide fundamental information on the stress response in these marine mammals, which may differ significantly from that of the terrestrial mammals from which most of our understanding is based. The

incidental finding of the impact of MegAce on the dolphin endocrine system has broad-scale implications for the welfare of dolphins under human care.

RELATED PROJECTS

Project: Pathophysiology of Stress in Wild and Managed-Care Bottlenose Dolphins
PI: Pat Fair
This project looks at numerous markers of stress in a wild population of marine mammals and compares them to animals under managed care in order to quantify and qualify the impact of environmental stressors on wild dolphins. The dolphins under managed care are from the Georgia Aquarium and the Navy Marine Mammal Program. A subset of the dolphins used in the current study (PI – Houser) are used as the semi-domesticated comparison.

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